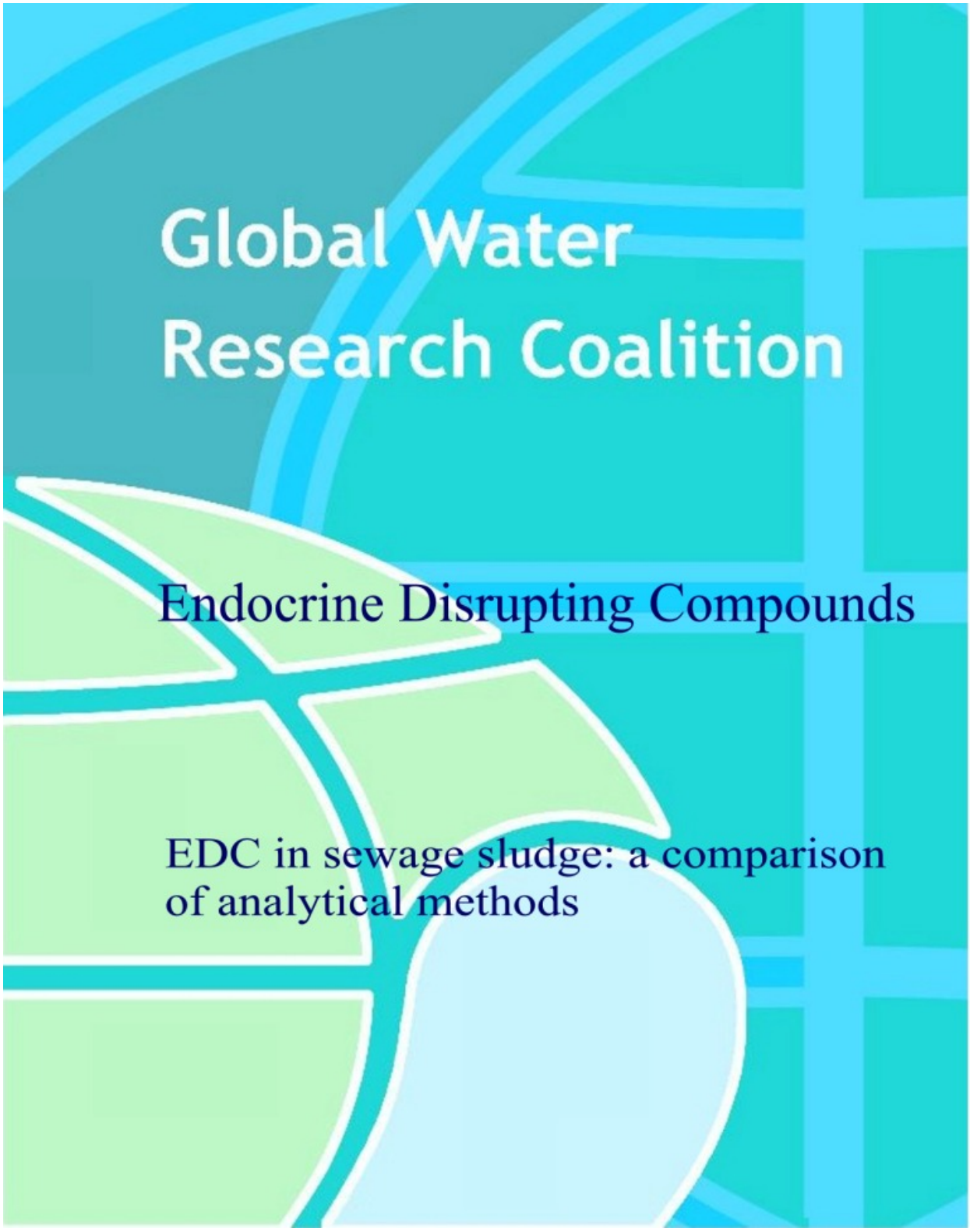
Global Water
Research Coalition
Endocrine Disrupting Compounds
EDC in sewage sludge: a comparison
of analytical methods

Global Water Research Coalition

IWA affiliate

Endocrine Disrupting Compounds

EDC in sewage sludge: a comparison of analytical methods

Prepared by:

UK Water Industry Research and Water Environment Research Foundation

February 2004

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Global Water Research Coalition

Global cooperation for the generation of water knowledge

GWRC is a non-profit organization that serves as a collaborative mechanism for water research. The benefits that the GWRC offers its members are water research information and knowledge. The Coalition focuses on water supply and wastewater issues and renewable water resources: the urban water cycle.

The members of the GWRC are: the Awwa Research Foundation (US), CRC Water Quality and Treatment (Australia), EAWAG (Switzerland), Kiwa (Netherlands), Suez Environment-CIRSEE (France), Stowa - Foundation for Applied Water Research (Netherlands), DVGW – TZW Water Technology Center (Germany), UK Water Industry Research (UK), Veolia- Anjou Recherché (France), Water Environment Research Foundation (US), Water Research Commission (South Africa), WateReuse Foundation (US), and the Water Services Association of Australia.

These organizations have national research programs addressing different parts of the water cycle. They provide the impetus, credibility, and funding for the GWRC. Each member brings a unique set of skills and knowledge to the Coalition. Through its member organizations GWRC represents the interests and needs of 500 million consumers.

GWRC was officially formed in April 2002 with the signing of a partnership agreement at the International Water Association 3rd World Water Congress in Melbourne. A partnership agreement was signed with the U.S. Environmental Protection Agency in July 2003. GWRC is affiliated with the International Water Association (IWA).

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Preface

Endocrine Disrupting Compounds (EDC) is one of the priority issues of the research agenda of the Global Water Research Coalition.

Analytical methods to determine the occurrence and fate of EDC in the water cycle are of vital importance to study the impact of EDC on public health and the aquatic environment. The aim of this specific research was to determine endocrine disrupting compounds (EDCs) in a selection of sewage sludges and soils using a newly developed analytical procedure (GWRC report EDC in sewage sludge – analytical method development (2003)) to determine free steroid oestrogens. The results of the chemical analysis were also to be compared with those from oestrogen bioassays.

This study was co-funded by the UK Water Industry Research (UKWIR) and the Water Environment Research Foundation (WERF). The GWRC likes to acknowledge UKWIR and WERF for their joint leadership to organise this study.

ENDOCRINE DISRUPTERS IN SEWAGE SLUDGE: A COMPARISON OF ANALYTICAL METHODS

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Report Title	**Endocrine Disrupters in Sewage Sludge: A Comparison of Analytical Methods**
Project Manager	**Gordon Wheale**
Collaborator	**WERF**
Contractor	**WRc-NSF Limited**
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Author of Report	**R Kanda**
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ENDOCRINE DISRUPTERS IN SEWAGE SLUDGE : A COMPARISON OF ANALYTICAL METHODS

Executive Summary

The aim of this research was to determine endocrine disrupting compounds (EDCs) in a selection of sewage sludges and soils using a newly developed analytical procedure (UKWIR report 03/TX/04/7) to determine free steroid oestrogens. The results of the chemical analysis were also to be compared with those from oestrogen bioassays. A further aim of this work was to fractionate solvent extracts of sewage sludge to isolate biologically active oestrogenic chemicals.

- Chemical analysis of soil and sludge samples showed that NPEOs and NPs were detected in all samples.

- Steroid oestrogens were not detected in any of the soil samples and ethinyl oestradiol was not detected in any of the sewage sludge samples. Oestrone was detected in activated sludge, mesophillic anaerobic sludge and heat dried sludge at low concentrations. 17-β-oestradiol was detected only in activated sludge and mesophilic sludge. Steroid conjugates were not detected in any of the sludge or soil samples.

- The environmental impact of the presence of very low concentrations of endocrine disrupters in sewage sludge on the terrestrial environment is unknown at present.

The oestrogenicity of sewage sludge and soils was determined using the recombinant yeast screen (YES) and the ER-CALUX bioassays. The results obtained from the YES bioassay were inconsistent and show poor correlation in relation to accuracy and recovery for spiked samples. Results obtained from the ER-CALUX bioassay showed that sewage sludge is oestrogenic. However, aqueous leachates of the soil and most of the sludge samples (except primary and activated sludge) gave no oestrogenic response. This suggests that the oestrogenic compounds present in sludge may not leach into groundwater or be bioavailable when applied to land.

Fractionation of solvent extracts of soil and sludge samples showed that in general the majority of oestrogenic activity was associated with two out of five HPLC fractions (F2 and F3). Chemical analysis of fraction F3 showed that it contained free steroid oestrogens and nonylphenols. The other four fractions including F2 were not subject to detailed chemical analysis.

The extraction and fractionation methods developed in this work need to be performance tested before any further conclusions can be drawn regarding the oestrogenicity of sewage sludge or in attempting to relate the oestrogenicity to the free steroids detected.

GLOSSARY

YES Bioassay recombinant yeast screen oestrogenicity bioassay

ER-CALUX Bioassay estrogenic receptor chemically activated luciferase gene expression bioassay

GCMS Gas chromatography mass spectrometry

LCMS Liquid Chromatography mass spectrometry

HPLC High Performance Liquid Chromatography

PSS Primary sedimentation sludge

AS Activated sludge

MAD Mesophilic Digested Sludge

HDS Heat dried sludge

LS Loamy soil

SIM Selected ion monitoring mode

ASE Accelerated solvent extraction

hER Human Estrogen receptor

EEQ Oestrogen equivalents (measure of oestrogenicity related to 17β-oestradiol)

WwTW Wastewater treatment works

SIM Selected ion monitoring

ASE Accelerated solvent extraction

SPE Solid phase extraction

LOD Limit of detection

ESI Electrospray ionisation

APCI Atmospheric Pressure chemical ionisation

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1 Introduction

Research carried out to date has shown that the natural steroid oestrogens, oestrone and 17-β-oestradiol and the synthetic steroid ethinyl oestradiol are the most oestrogenically active chemicals found in sewage effluents. Other compounds detected in sewage effluents including some environmental contaminants, agricultural and industrial chemicals have also been implicated as potential endocrine disrupters. Evidence for the oestrogenic activity of such effluents comes from the UK (Purdom *et al.*,1994, Montaganani *et al.*,1996, Harries *et al.*,1997)[1] [2] [3] , Germany (Stumpf *et al.*,1996, Ternes *et al.*,1999) [4] [5], Italy (Johnson *et al.*,2000) [6], the Netherlands (Baronti *et al.*,2000) [7], Sweden (Larsson *et al.*,1999) [8] and the USA (Snyder *et al.*,1999) [9].

Recent work carried out to model the fate of steroid oestrogens (SOs) during sewage treatment has indicated that some will partition in significant amounts onto sewage sludge (UKWIR, 2000).

1.1 Project Objectives

The specific objectives of this work programme were:

1. To apply a newly developed analytical method (UKWIR 2003) [11] to measure free steroid oestrogens and to determine the concentration of nonylphenols and nonylphenol ethoxylates in a selection of sewage sludges and soils.

2. To determine the total oestrogenicity of a range of sludges using selected oestrogen bioassays

3. To fractionate sewage sludge samples to isolate chemicals which are biologically active

1.1 Chemical methods of analysis

The analytical methodology used to determine nonylphenols, nonylphenol ethoxylates in sludges and soils are given in the appendices. The analytical methodology used to determine free steroid oestrogens has been previously described (UKWIR 2003) [11].

1.2 Biological screening methods

A number of biological screening methods or bioassays are available for the determination of oestrogenic activity of individual chemicals or environmental samples. The most widely used is based on the human breast tumour cell line, MCF-7. In this (E-SCREEN) bioassay MCF-7 cells are grown in a culture. The presence of an oestrogen results in the proliferation of the cells whilst an absence of an oestrogen results in the prevention of proliferation. The MCF-7 is a relatively simple and robust test and has been widely applied. The E-SCREEN is a very sensitive assay which can be used to measure the effects of single chemicals or complex mixtures (Soto *et al.* 1995)[12]. Although cell proliferation assays have their limitations (Zacharewski 1997)[13] they can provide quantitative estimates and are applicable to environmental samples (Korner *et al* 1999)[14].

Other commonly used bioassays are the ER-CALUX (estrogenic receptor chemically activated luciferase gene expression) available from BioDetection Systems b.v. Amsterdam (Legler 1999) and yeast bioassays (Routledge and Sumpter 1996, Rehmann *et al*. 1999).

The ER-CALUX assay is an *in vitro* bioassay carried out with a recombinant human T47D breast adenocarcinoma cell line which contains original oestrogen receptor (ER) and includes a stable ER-mediated firefly (*Photinus pyralis*) luciferase gene expression. When oestrogenic compounds are brought into contact with the cell, they pass through the cell wall and bind and activate the human oestrogenic receptor. Consequently, luciferase is formed in the cell and its quantity is a direct measure of the oestrogenic potential of the substance or the extract being studied. The amount of light emitted, which is proportional to the quantity of luciferase, can be measured by a luminometer. The quantity of light emitted is interpolated in the calibration curve for 17β-oestradiol and is reported in terms of 17β-oestradiol equivalents (ng l^{-1} of EEQ)[18].

Seung-Min *et al.* (2000)[19] used an optimised E-SCREEN for the sensitive quantitative determination of total oestrogenicity in river water and sediment samples in Korea. The detection limit in terms of 17β-oestradiol equivalent concentration of the E-SCREEN assay was 8.0 pg EEQ l^{-1}. The total oestrogenic activity in the river water samples was between 0.5 pg l^{-1} and 7.4 ng l^{-1}, while for the sediment extracts the range was 3.4 to 10.7 pg g^{-1}.

The recombinant yeast screen oestrogenicity bioassay (YES) is based on a similar concept to the E-SCREEN but is based on yeast constructs expressing a human oestrogen receptor. The reporter gene is *lac*-Z, encoding for the enzyme β-galactosidase. Hence, in the presence of oestrogenic compounds, β-galactosidase is synthesised and secreted into a medium which contains the chromogenic substrate chlorophenol red-β-galactopyranoside (CPRG). The enzyme metabolises CPRG, which is initially yellow into chlorophenol-red which is a red product. The absorbance of the red colour is determined spectrophotometrically and compared to the absorbance of wells containing standard solutions of β-oestradiol to determine the oestrogenic potency of the sample/substrate under investigation.

The YES bioassay is extensively used world-wide in screening chemicals and environmental samples for oestrogenicity. The widespread use of this assay is due to the yeasts used in the assay being easy to manipulate and grow. The assay also allows the rapid screening of numerous chemicals or samples to be performed over a wide dose-response range in a short timeframe.

Desbrow *et al.* (1998)[20] applied the YES bioassay to isolate the major oestrogenic chemicals present in sewage treatment works effluents. Witters *et al.* (2001)[21] used a YES assay to evaluate the oestrogenic activity of extracts prepared from 16 samples taken in Flemish rivers and reservoirs for drinking water production or effluents from municipal wastewater treatment plants. Oestrogenic potency in water samples ranged from below the detection limit (~2.75ng l^{-1} EEQ) up to 81 ng l^{-1} EEQ).

Murk *et al.* (2002)[22] used three *in vitro* bioassays to determine the oestrogenic potency of wastewater and surface water samples taken in the Netherlands. The oestrogenicity of extracts from sludge samples determined using the ER-CALUX and YES bioassays were 1.6 to 41 and <dl to 13 pmol/g EEQ. Work carried out by Korner *et al.* (2000)[23] using the E-Screen assay has shown that sewage sludge collected from Steinhaule, Germany had an oestrogenic activity of 1.6 ng g^{-1} EEQ in raw secondary sludge and 3.7 ng g^{-1} EEQ in dried primary and secondary sludge.

There are a number of drawbacks in using any *in vitro* bioassay for the determination of oestrogenicity of sewage samples. When using the YES bioassay Desbrow *et al.* (1998)[20]

found that testing of whole effluent samples did not elicit a response unless the samples were sterilised using membrane filtration prior to the assay. The researchers attributed this problem to the growth of bacteria present in the samples competing with the yeast growth. The same authors have also warned of the suitability of the assay if the extracts contain high levels of toxic substances, due to toxicity to the yeast cells. The USEPA have also reported that the YES assay cannot detect the estrogenic activity of chlorinated chemicals (USEPA, 1997)[24].

A further major problem with this and other *in vitro* bioassays is that anti-oestrogens present in the sample can antagonise the oestrogen receptor and reduce the oestrogenic response. Therefore, false negatives or an underestimation of oestrogenic response can occur. Another issue with *in vitro* bioassays is that different studies have reported different oestrogenic potency for individual compounds using the same bioassay. Several *in vitro* bioassays have reported the oestrogenicity of 17β-oestradiol and ethinyl oestradiol as equivalent whilst other studies have shown ethinyl oestradiol to be ten times as potent (Winter-Nielsen M and Helweg C., 2002)[25].

The YES bioassay was selected to carry out oestrogenic bioassay screening of sewage sludge samples as this bioassay is readily available and is commonly used in the UK to determine oestrogenic potency of sewage effluent samples. Due to problems with spiked control samples during the method development stage, further work was carried out using the ER-CALUX bioassay.

2 Sampling and Chemical Analysis

Samples were obtained from a sewage treatment works in Southern England. The works has a primarily domestic input and serves a population of 130,000 (flow 62 Ml/day); it is equipped with a standard mesophilic anaerobic digester and a sludge drier.

The following samples were collected:

1. Primary sedimentation sludge

2. Activated sludge

3. Mesophilic anaerobic digested sludge

4. Heat dried sludge

5. Loamy soil (Kettering loam)

The loamy soil selected for this work was Kettering loam (from Turf Management Systems, Buckinghamshire, UK) which has previously been used as a standard test soil in a series of national and international projects on terrestrial ecotoxicology.

All sludge samples were collected in pre-washed glass bottles with glass stoppers. Samples were delivered to the laboratory within 1 day of collection. The samples were kept cold (2-6 °C) during transportation in a cool box, and on arrival at the analytical laboratory stored in a fridge at 2-6 °C prior to analysis.

The samples collected contained the following amounts of solids: Primary sedimentation sludge (PSS) 3-4%, Activated sludge (AS) 0.5%, Digested Sludge (MAD) 2-3%, Heat dried sludge (HDS) >95% and Loamy soil (LS) >95%.

Sludge and soil samples received in the analytical laboratory were categorised into two groups; solids (loamy soil, heat dried soil) and slurries (primary sludge, activated sludge and digested sludge). The slurry samples were centrifuged to separate the solids from the aqueous phase.

The solid phases were freeze dried prior to extraction and analysis for free steroids, alkylphenol ethoxylates and alkylphenols. The analytical methodologies used are described in the appendices.

The results obtained are shown in. The data obtained show that nonylphenol ethoxylates were detected in all sludge and soil samples. Nonylphenols were also detected in all soil and sludge samples. High levels of nonylphenols in mesophilic anaerobic sludge suggest that alkylphenols are formed and are persistent under these conditions.

Oestrone and oestradiol were detected in all of the sludge samples at low levels. Ethinyl oestradiol concentrations were generally below the limit of detection. The data obtained is from a single sewage treatment works but shows that steroid oestrogens are only present in sewage sludge at low levels.

Steroid conjugates were not detected in any of the soil or sludge samples analysed. This included samples that had been spiked with steroid conjugates as positive controls with the exception of the quality control sample (steroids spiked into sand) which showed a recovery of 50-60% of the steroid conjugates.

The work carried out suggests that the steroid conjugates are readily cleaved, possibly into free steroids, in samples which exhibit enzymatic activity. Further work is required to establish more reliable analytical methodology for the determination of steroid conjugates.

Table 2.1 Chemical analysis of sewage sludge and soils for endocrine disrupting compounds

Sample	Concentration ng g^{-1}							
	Nonylphenol ethoxylates	**Nonylphenols**	**Ethinyl oestradiol n=11**	**17-β-oestradiol (std dev) n=11**	**Oestrone (std dev) n=11**	**Oestrone-3-sulfate**	**Oestradiol-3,17-disulfate**	**Oestradiol-3-glucoranide-17-sulphate**
Loamy soil	8.1	0.4	<25	<25	<25	<100	<100	<100
Primary sludge	11.7	30.3	<100	<100	<100	<100	<100	<100
Activated sludge	51.4	19.7	<25	35 (9.4)	60 (14.5)	<100	<100	<100
Mesophilic anaerobic sludge	21.9	114	<25	27 (6.8)	44 (8.8)	<100	<100	<100
Heat dried sludge	27.5	8.7	<25	<25	48 (14.3)	<100	<100	<100

3 Oestrogenicity of sewage sludge and soil

3.1 Determination of total oestrogenicity of sewage sludge and soil

The aim of these experiments was to determine the total oestrogenicity of sewage sludge and soil samples. Initial method development (Experiment 1) was undertaken on samples collected from a WwTW in Southern England in October 2002.

Further samples were collected in January 2003 for a repeat of the work undertaken in October 2002 and for further analysis to optimise the extraction solvent used (Experiment 2).

3.1.1 Experiment 1: The determination of total oestrogenicity of sewage sludge and soils using dichloromethane:acetone (1:1)

Sludge and soils samples received in the analytical laboratory were categorised into two groups, the first were classified as solids (loamy soil and heat dried sludge) and the second group as slurries (primary sludge, activated sludge and digested sludge).

The slurry samples were centrifuged to separate the solids from the aqueous phase. The solid phases were freeze dried and a portion (5g) was extracted using dichloromethane/acetone (1:1) using accelerated solvent extraction (ASE).

ASE extraction was carried out based on a procedure developed by Richter *et al.*, (1994) for the extraction of bases, neutrals and acids, which meets the sample extraction requirements of USEPA method 3545. The ASE extraction conditions used are provided in Table 3.1. A positive control sample was extracted alongside the sewage sludge and soil samples. This control was loamy soil which was spiked with a standard mixture of oestrone, 17-β-oestradiol and ethinyl oestradiol at 100 ng g^{-1} of each individual steroid.

The extract was concentrated to 1 ml using a TurboVap™ concentrator and then split into two equal portions of 0.5 ml. The first aliquot was reduced to dryness and diluted by a factor of 10 using methanol prior to analysis using the YES Bioassay. The remaining fraction was also reduced to dryness and reconstituted into methanol. (0.1 ml) and fractionated using HPLC (see section 3.3).

The YES bioassay of the extract was undertaken by CEFAS (Burnham Laboratory, UK). The assay was carried out using the method developed by Routledge and Sumpter (1996). Known amounts of 17β-oestradiol (calibration standards) and sample extracts were added to microtitre plates, followed by media containing the yeast cell suspensions and the yellow substrate chlorophenol red-β-galactopyranoside (CPRG). The plates were mixed by agitation and incubated at 32 °C under humid conditions for 3-4 days.

Table 3.1 Accelerated solvent extraction conditions for extraction of sewage sludge and soils for oestrogenicity bioassays

Equipment	ASE 200 Accelerated solvent extractor with 33 ml stainless steel cells
Oven Temperature	150°C
Pressure	14 MPa (2000 psi)
Oven Heat-up time	5 minutes
Flush Volume	60% of extraction cell volume
Solvent	Dichloromethane:acetone (experiment 1) or methanol (experiment 2)
Nitrogen Purge	1 MPa (150 psi) for 60 seconds

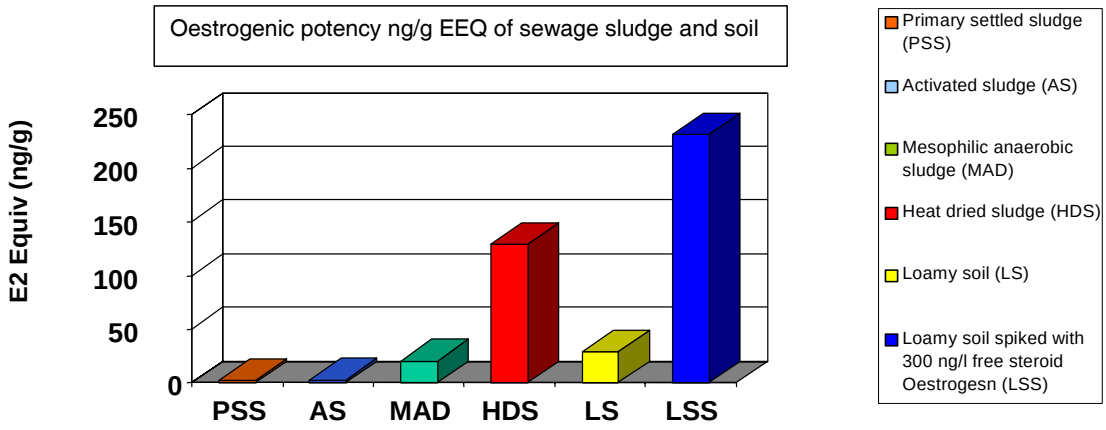
At least three dilutions of the sample extract were tested to ensure that quantitation was performed at concentrations within the range of the calibration curve and to ensure that quantitation was not performed where there was inhibition of the signal due to the toxicity of the extract to the yeast.

The absorbance (at 520 nm) of the individual wells was read spectrophotometrically using a microplate reader. The absorbance of individual wells was compared to the 17β-oestradiol calibration curve to determine oestradiol equivalent (EEQ) concentrations in the sample.

The total oestrogenicity of sewage sludge extracts (solvent extracted using 1:1 dichloromethane:acetone) and a loamy soil (unspiked and spiked with steroid oestrogens at 300 ng g^{-1} total) is shown in Table 3.2 (page 11) and Figure 3.1.

Figure 3.1 Total oestrogenicity of solvent extracts of sewage sludge and soil samples determined using the YES Bioassay

These initial results show that sewage sludges from the WwTW studied exhibit between 2 to



129 ng g^{-1} EEQ oestrogenic potency (detected by the YES bioassay). The total oestrogenicity of heat dried sludge was very high (129 ng g^{-1}). The positive control spiked at 300 ng g^{-1} gave a result of 231 ng g^{-1}. Unfortunately a negative control (e.g. baked salt) was not submitted for the assay as it was assumed that the loamy soil would act as a blank. However, the unspiked soil sample showed an oestrogenic activity of 28 ng g^{-1} EEQ. Although there is very little data on oestrogenicity of soils in the literature, this value was considerably higher than expected. This data therefore raised questions about the reliability of the YES bioassay and the extraction procedure used.

It was therefore decided that further work was required to determine the reproducibility of both the extraction (ASE) method and the YES oestrogen screen.

3.1.2 Experiment 2: The determination of the reproducibility of the YES screen and optimisation of extraction solvent

Further sludge samples were collected in January 2003. Sampling and sample storage was carried out in an identical way to the samples collected in October 2002. The slurry samples were also centrifuged using identical conditions to those used in Experiment 1.

One portion (5g) of the solids was extracted using dichloromethane/acetone (1:1) with the ASE conditions used in experiment 1. The second portion of the sample was extracted using methanol using the ASE conditions shown in Table 3.1. In addition to the samples extracted in Experiment 1, a number of additional positive and negative controls were included in this experiment to detect any false positives or false negatives.

An OECD synthetic soil often used in ecotoxicological testing work at WRc was introduced as a control sample. One aliquot was extracted unspiked (negative control) and another spiked (positive control) with 250 ng g^{-1} of 17β-oestradiol. In addition, sodium sulphate salt (baked in a furnace overnight at 600°C) was also extracted and used as a method blank.

Data generated using OECD soil spiked with 250 ng g^{-1} of oestradiol (positive control) and gave a result of 187 ng g^{-1} EEQ (using the YES bioassay). Furthermore, the furnace baked sodium sulphate (negative control) and the blank OECD synthetic soil contained <0.17 and 0.5 ng g^{-1} EEQ dry weight respectively indicating that the new methodology was fit for purpose.

The results obtained from the DCM:acetone extracts were unexpectedly different and markedly lower and below the limit of detection of 0.17 ng g^{-1} EEQ for all samples tested except for mesophilic anaerobic sludge (Table 3.2). Considering that all three controls (positive and negative) gave expected results, it can be inferred that the samples were not oestrogenic or that there were problems with the bioassay possibly due to the toxicity of the solvent extracts.

Other work recently carried out on the oestrogenicity of solid samples (not published or presented here) has shown that the YES bioassay of methanol extracts provides better reproducibility. The work carried out here using methanol as the extraction solvent also supports this view. **Therefore the data produced in Experiment 1 should be ignored**.

The oestrogenic potency of sludge and soil samples obtained from methanol extracts of primary sedimentation sludge (PSS), activated sludge (AS), heat dried sludge (HDS), mesophilic anaerobic digested sludge (MAD) using the YES bioassay were 5.7, <0.17, 27

and 15 ng g^{-1} EEQ dry weight respectively. The oestrogenicity of loamy soil was found to be <0.17 ng g^{-1} EEQ dry weight (Table 3.2).

The YES bioassay data for activated sludge using methanol and DCM:acetone for extraction appears to show that activated sludge is not oestrogenic or it could contain interferences within the sample (e.g. anti-oestrogens) that antagonise the hER, resulting in a low response.

These extracted samples were therefore submitted for oestrogenicity and anti-oestrogenicity determination using the YES bioassay. The repeat analysis of both the methanol and DCM extracts showed that the oestrogenic activity of sludge extracts was <21 ng g^{-1} and that there was no anti-oestrogenic activity (<400 ng tamoxifen g^{-1}) which would indicate that these extracts were not oestrogenic (Table 3.3).

As a further check to determine the true oestrogenicity of soils and sewage sludge, the methanol extracts were reduced to dryness and re-dissolved in dimethyl sulfoxide (DMSO) and the extracts were sent to BioDetection Systems B.V., (Amsterdam) for oestrogenicity determination using the ER-CALUX™ bioassay. The results obtained were compared with the data from the YES bioassay and the results are shown in Figure 3.2.

The data obtained from methanol extracts of soil and sewage sludge using the YES and ER-CALUX bioassay show that sewage sludge is oestrogenic. However, further work is required to performance test the methods used prior to drawing any conclusions regarding from the data obtained.

Figure 3.2 Oestrogenic activity of methanol extracts of loamy soil and sewage sludge from various collection points within a sewage treatment works

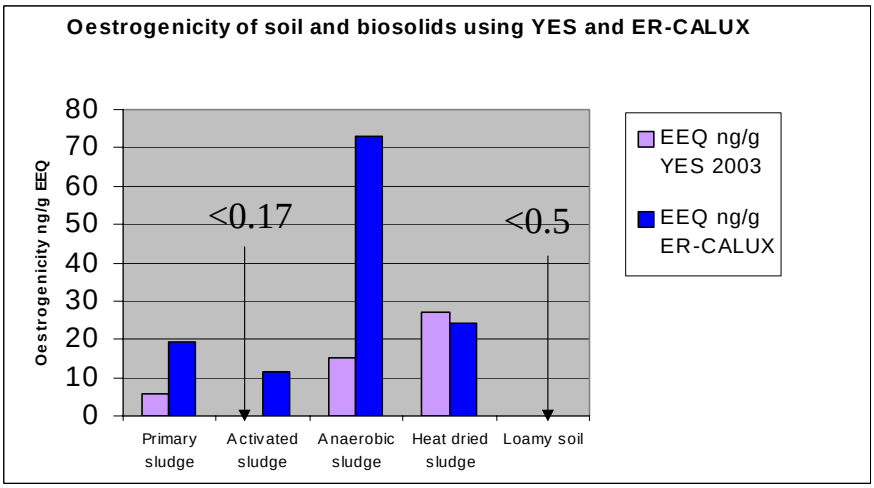


Table 3.2 Oestrogenicity of sewage sludge and soils determined using different extraction procedures. Samples were extracted with either dichloromethane:Acetone or methanol or using an aqueous extraction based on USEPA method 1311 (Toxicity Characteristic Leaching Procedure). Oestrogenicity was determined using the YES bioassay.

Sample	DCM:Acetone 2002	DCM:acetone 2003	Methanol 2003	Aqueous leachate##	
				EEQ ng L^{-1}	EEQ in ug/Kg of solids
	µg E2 Equivalents Kg^{-1} (St. Dev.) n=5			(St. Dev.) n=5	
Activated Sludge	2.6	<0.17	<0.17	68 (11)	1.36
Primary Sludge	2	<0.17	5.7 (2.9)	115 (12)	2.3
Heat dried sludge	129.1	<0.17	27 (8)	<21	<0.42
Loamy soil	28.7	<0.17	<0.17	<21	<0.42
Mesophilic anaerobic digested sludge	20.4	53 (10)	15 (9)	<21	<0.42
OECD Blank soil	NR	<0.17	0.5 (0.08)	<21	<0.42
OECD oestradiol spiked soil*	NR	161 (20)	187 (16)	<21	<0.42
Sodium sulphate blank	NR	<0.17	<0.17	<21	<0.42
Loamy spiked soil#	231	NR	NR		
Liquids (note d)					
Activated sludge liquid fraction				<21	
Primary sludge liquid fraction				<21	
Activated sludge liquid fraction (C18				27 (7)	
Primary sludge liquid fraction (C18				43 (9)	
Liquids spiked (note d)					
Activated sludge liquid fraction (spiked)**				92 (23)	
Primary sludge liquid fraction (spiked)**				<21	

*OECD Soil was spiked at 250 ng/g oestradiol #Loamy soil was spiked with a mixture of steroid oestrogens (300 ng/g) ** Liquid samples were spiked with 250 ng/l oestradiol # # Extracted using a method based USEPA method 1311

3.1.3 Experiment 3: Determination of the oestrogenicity of the liquid fraction of sewage sludge

Primary and activated sludge samples were centrifuged and the liquid samples were submitted for YES oestrogen bioassay. As positive controls, portions of the sludge liquids were spiked with 250 ng l^{-1} of 17β-oestradiol. The approach was based on the findings of the COMPREHEND (Community programme of research on environmental hormones and endocrine disrupters) project, *viz* that the YES assay was suitable for the direct screening of unfractionated sewage influents.

Additionally, aliquots of the liquid samples were extracted using C18 solid phase extraction using the approach of Desbrow *et.al.* to determine oestrogenicity of effluents. This was carried out to compare the oestrogenic activity data with that obtained from literature values for sewage effluents.

The data obtained from C18 SPE extracts of the liquid portion of the sludge sample showed that activated and primary sludge contained 27 and 43 ng l^{-1} EEQ of oestrogenic activity. The results therefore suggest that oestrogenic compounds are present in the liquid phase of sewage sludge (Table 3.2). However, the results obtained from whole samples (unfractionated) were unexpected (Table 3.2) as they were low (and lower than the same samples extracted using a C18 cartridge). In addition the liquid from the primary sludge sample which had been spiked with 17β-oestradiol did not produce a response from the YES bioassay. This suggests that either the 17β-oestradiol added to the sample had been metabolised by biological or enzymatic activity or that the liquid contains other interferences (e.g. anti-oestrogens) that antagonise the hER resulting in false negatives.

As a further check of the data obtained liquid samples of activated sludge were spiked at two levels (500 ng l^{-1} and 1000 ng l^{-1}) and were submitted along with one unspiked sample for oestrogen and anti-oestrogen analysis using the YES and ER-CALUX bioassay. The results obtained are shown in Table 3.3. The data clearly show that there is excellent correlation between the data obtained from ER-CALUX and very poor correlation with data obtained using the YES bioassay.

Further work was carried out by Biodetection systems on repeat analysis of the spiked and unspiked samples and on the whole effluent and hexane:dichloromethane (1:4 v/v) extracts of the samples. The results are shown in Table 3.4, which again show the excellent reproducibility of the ER-CALUX methodology. The results obtained using this methodolgy show that the liquid phase of activated sludge is oestrogenic (87 ng l^{-1} EEQ).

Table 3.3 Oestrogenic activity associated with the liquid and solid phase of activated sludge determined using the YES and ER-CALUX bioassay

Sample	WRc-NSF spike (17β-oestradiol)	YES Bioassay		ER-CALUX Bioassay	
		Oestrogenic Activity (St. Dev)	Anti-Oestrogenic Activity	Oestrogenic Activity (St. Dev)	Anti-Oestrogenic Activity
Whole water samples	ng l^{-1}	ng l^{-1} EEQ	ng tamoxifen l^{-1}	ng l^{-1} EEQ	ng tamoxifen g^{-1}
Activated sludge – A	**Not spiked**	**<5**	<100	**87 (4.5)**	NR
Activated sludge – B	**500**	**11 (4)**	<100	**511 (39.3)**	NR
Activated sludge – C	**1000**	**15 (6)**	<100	**1063 (117)**	NR
Sludge Solvent Extracts		ng l^{-1} EEQ	ng tamoxifen g^{-1}	ng l^{-1} EEQ	ng tamoxifen g^{-1}
Activated sludge methanol extracted	Not spiked	<21	<400	7.3	ND
Activated sludge DCM extracted	Not spiked	<21	<400	11.5	ND

ND=Not detected; NR=Not reported

Table 3.4 ER-CALUX bioassay showing repeatability of assay and comparison of oestrogenicity of whole effluent and solvent extract

Sample code	WRc-NSF spike	Whole effluent screening		Whole effluent re-analysis		Solvent extracts	
	ng/l EEQ	ng/l EEQ	SD (%)	ng/l EEQ	SD (%)	ng/l EEQ	SD (%)
A	Unspiked	87	5.2	62	4.7	26	7.0
B	500	511	7.7	562	9.1	348	6.0
C	1000	1063	11	1020	4.1	716	5.9

3.2 Determination of bioavailable oestrogenicity of sewage sludge and soil

The data from solvent extracts represent the maximum total oestrogenicity in the sludge and does not provide any information on what portion of this total oestrogenicity is bioavailable.

Solid soil and sludge samples were therefore extracted using water to determine the amount present in the sample, which would be bioavailable. The extraction procedure used was based on USEPA Method 1311 (Toxicity Characteristic Leaching Procedure). This procedure is designed to determine the mobility of both organic and inorganic analytes present in liquid, solid and multiphasic wastes.

Solid samples containing a liquid phase were centrifuged as described above and the solid portion was extracted using USEPA method 1311.

Prior to extraction of the sample, two extraction fluids were prepared. Fluid 1 was made with 5.7 ml of 5% glacial acetic acid in 500 ml reagent water and 64.3 ml of 1N sodium hydroxide. The mixture was diluted to 1 litre and the pH checked to ensure that it was 4.93. Fluid 2 was made by diluting 5.7 ml of 5% glacial acetic acid in 1 litre of reagent water litre and the pH checked to ensure that it was 2.88.

The extraction fluid used for extraction of each of the solid samples was determined by weighing a small amount of the sample into a beaker and adding reagent water. The sample was stirred vigorously for 5 minutes using a magnetic stirrer. After stirring, the pH of the liquid was determined. For samples with a pH <5.0 fluid 1 was used for extraction. For samples where the pH was >5 fluid 2 was used.

A 5g portion of the solid sample was added to an extractor vessel and extracted with 100 ml (solid to extraction fluid ratio of 20) of either fluid 1 or 2 determined from the fluid selection exercise above.

The sealed extractor bottle was agitated on a shaker for 18 hours at ambient temperature (23°C). Following extraction, the liquids were separated using a centrifuge. Samples were not filtered in case there was adsorption of EDCs on to the filter.

Most leachates gave no response to the YES bioassay, although primary and activated sludge gave results of 115 and 68 ng l^{-1} EEQ respectively (Table 3.2). Considering that 5 g of the sample was extracted into 100 ml of liquid this equates to an activity in the original solids of 1.36 and 2.3 ng g^{-1} EEQ. In similar work carried out in Germany in which sewage sludge was applied to sandy soil in lysimeters, few leachate samples induced growth of human breast cells in the E-Screen.

3.3 Determination of the Oestrogenicity of HPLC Fractions

Research has shown that over 90% of the oestrogenic activity of sewage effluents is due to the presence of three free steroids oestrone, 17-β-oestradiol and ethinyl oestradiol. Other EDCs which have been implicated include nonylphenols, APEOs, phthalates, polybrominated diphenylethers, pesticides phthalates, Bisphenol A and a growing list of other compounds including pharmaceuticals and personal care products (PPCPs).

The aim of these experiments was to isolate HPLC fractions to determine how the total oestrogenicity obtained from solvent extracts of sludge relates to individual EDCs present in the sludge.

Sludge samples were solvent extracted as described under Experiment 1 above and reconstituted into methanol. The methanol extracts of sludge and soil samples were fractionated using HPLC and analysed using the YES bioassay to isolate compounds and/or fractions which were biologically active.

HPLC fractionation was carried out using a Hewlett Packard 1050 HPLC system which was equipped with a HP1050 quaternary pump, column heater and UV detector. The detector was connected to a Dynamax fraction collector (model FC-4). The extract was manually injected via a Rheodyne injector (100 μl loop). The fractions were separated on a Waters Spherisorb S5ODS1 (25cm x 4.6 mm id) column by use of a gradient. The initial mobile phase composition was 60% water and 40% methanol at a flow rate of 1 ml min^{-1}. A 30 minute linear gradient was used to reach a final composition of 100% methanol which was held for 10 minutes. The re-equilibration to initial conditions required 3 minutes.

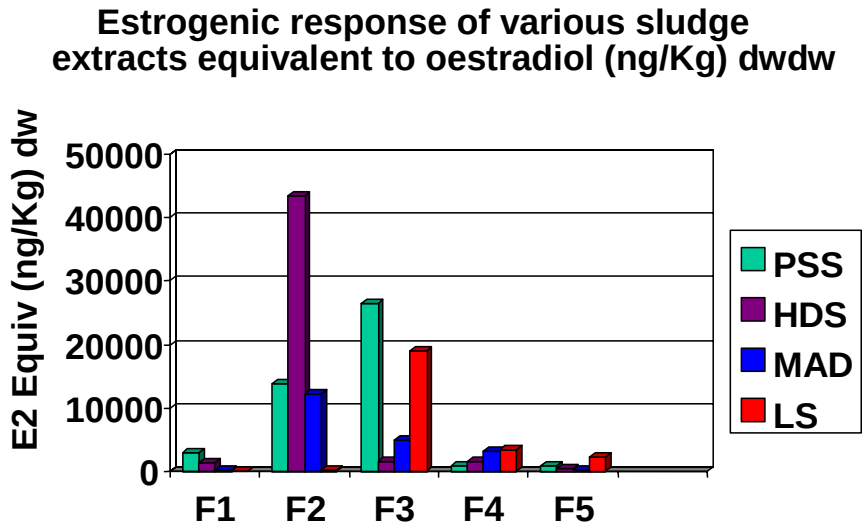
Fractions were collected every 7 minutes and each fraction was 7 ml in volume. Each fraction collected was concentrated to 1ml prior to analysis by the YES bioassay. The YES bioassay was carried out by CEFAS (Burnham Laboratory, UK).

The oestrogenicity of HPLC fractions of solvent extracted sludge and soil fractions is shown in Figure 3.3.

The results obtained show that the majority of the oestrogenicity of various sludges (with the exception of activated sludge) originates from fractions F2 and F3. Chemical analysis of fraction F3 showed that it contained free steroid oestrogens and nonylphenols. The other four fractions including F2 were not subject to detailed chemical analysis.

Further fractionation is required to identify compounds that cause oestrogenicity in fraction F2. Prior to carrying out this work it is essential that the procedure by which oestrogenicity is measured (both the extraction method and the bioassay itself) is rigorously checked to ensure that the conclusions arising from the data are meaningful.

Figure 3.3 Oestrogenicity of individual fractions (F1-F5) of various sludge and soil extracts



4 Conclusion

The aim of this research was to determine endocrine disrupting compounds (EDCs) in a selection of sewage sludges and soils. Established analytical methodology was used to determine nonylphenol ethoxylates and nonylphenols and a newly developed analytical procedure was used to determine free steroid oestrogens.

Analysis of soil and sludge samples showed that NPEO and NPs were detected in all samples. Steroid oestrogens were not detected in any of the soil samples and ethinyl oestradiol was not detected in any of the sewage sludge samples. Oestrone was detected at low levels in activated sludge, mesophillic anaerobic sludge and heat dried sludge. 17-β-oestradiol was detected only in activated sludge and mesophilic sludge. Steroid conjugates were not detected in any of the sludge or soil samples.

The oestrogenicity of sewage sludge and soils was determined using the recombinant yeast screen (YES) and the ER-CALUX bioassays. The results obtained from the YES bioassay were inconsistent and show poor correlation in relation to accuracy and recovery for spiked samples. Results obtained from the ER-CALUX bioassay showed that sewage sludge is oestrogenic. However, aqueous leachates of the soil and most of the sludge samples (except primary and activated sludge) gave no oestrogenic response. This suggests that the oestrogenic compounds present in sludge may not leach into groundwater or be bioavailable when applied to land.

Fractionation of solvent extracts of soil and sludge samples showed that in general the majority of oestrogenic activity was associated with two out of five HPLC fractions (F2 and F3). Chemical analysis of fraction F3 showed that it contained free steroid oestrogens and nonylphenols. The other four fractions including F2 were not subject to detailed chemical analysis.

The extraction and fractionation methods developed in this work need to be performance tested before any further conclusions can be drawn regarding the oestrogenicity of sewage sludge or in attempting to relate the oestrogenicity to the free steroids detected.

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APPENDIX A : Alkylphenol polyethoxylates (APEO)

Freeze dried samples (5g) were ground and mixed with an equal proportion of Hydromatrix™. The samples were placed into an ASE stainless steel extraction cell and the void at the top of the extraction cell was filled using more Hydromatrix™.

ASE extractions were carried out using methanol extraction at 100°C, 100 atm, in static mode using a procedure developed by Valsecchi *et.al* (2001)[1]. The extracts were concentrated to a final volume of 1 ml using a TurboVap™ concentrator and analysed using LCMS.

LCMS analysis was carried out using positive ion atmospheric pressure chemical ionisation in selected ion monitoring mode (SIM). An Agilent 1100 liquid chromatograph (Agilent, USA) connected to a Micromass Quattro LC mass spectrometer (Manchester, UK) was used.

LCMS analysis was carried out by installing the analytical column (Hypersil APS2 5µm 150 mm x 4.6 mm) and conditioning the column for an hour with the eluent at a flow rate of 0.5 ml per minute. After the conditioning period the LC was connected to the mass spectrometer and the source parameters were set to the conditions shown in Table A1

Table A1: LCMS conditions for APEO analysis

LC:	**HP 1100 Series**				
Column:	Hypersil APS2 5µm 150 mm x 4.6 mm				
Flow:	1 ml/min				
LC Initial:	Solvent A: 90% Hexane:dichloromethane (2:1) Solvent B: Acetonitrile:iso-propanol (3:1)				
LC Gradient:					
Time	0 min	2 min	12 min	20 min	30 min
%A	90%	10%	50%	50%	90%
Injection volume:	2 ul				
MS:	**Micromass Quattro LC**				
Source:	Atmospheric Pressure chemical ionisation (positive ion)				
Source Temp:	150°C				
Desolvation Temp:	400°C				
Corona voltage:	3.5 Kv				
Cone Voltage:	60 V				
Nebuliser:	150 litres/hour				
Drying:	750 litres/hour				

Ions monitored:	287.2, 293.2, 331.22, 337.20, 375.20, 419.27, 463.30, 507.30, 551.30, 595.38, 639.41, 683.43, 727.40, 771.40, 815.40, 859.40 and 903.3. All ions monitored $[M+Na]^+$

APPENDIX B : Nonylphenols (NP)

Freeze dried samples (5g) were ground and mixed with an equal proportion of Hydromatrix™. A mixture of labelled internal standards was added to compensate for extraction efficiency. The samples were placed into an ASE stainless steel extraction cell and the void at the top of the extraction cell was filled using more Hydromatrix™.

ASE extractions were carried out using dichloromethane extraction at 100°C, 150 atm, in static mode using a procedure. The extracts were concentrated to a final volume of 1 ml using a TurboVap™ concentrator and analysed using GCMS.

GCMS analysis was operated in the positive ion electron mode using selected ion monitoring. An Hewlett Packard 5890 gas chromatograph (Agilent, USA) connected to a VG Trio-1 mass spectrometer (Manchester, UK) was used.

GCMS analysis was carried out using a J&W DB-5 30 m x 0.25 mm capillary column. The GCMS conditions used are shown in Table A2.

Table A2: GCMS conditions for alkylphenol analysis

GC:	**HP 5890**
Injector:	Cool on-column
Column:	J&W DB-5 30 m x 0.25 mm
Flow:	1 ml/min
Temp programme:	40°C hold for 4 minutes, to 250°C at 8°C/min, to 300°C at 16°C/min
Injection volume:	1 μl
MS:	**VG Trio-1**
Source:	Electron impact (positive ion)
Source Temp:	250°C
Interface Temp:	250°C
Ions monitored:	121, 135, 107, 188 (Internal standard)

APPENDIX C : Steroid conjugates

The objective of this work was to determine if steroid conjugates were present in sewage sludge and soils.

There are no published methods on the determination of steroid conjugates from sewage sludge although some non performance tested methods are available for the detection of some conjugates in aqueous samples.

Due to the very hydrophilic nature of steroid conjugates it was decided that sludge and soil samples would be extracted using ultrasonication in water. As positive controls all sludge and soil samples under investigation were spiked with a range of steroid conjugates.

Freeze dried sludge and soil samples were stored overnight at room temperature alongside samples spiked with steroid conjugates (oestrone-3-sulfate, oestradiol-3,17-disulfate and oestradiol-3-glucoranide-17sulphate). A further positive control (steroid conjugates spiked into furnace baked sand) was analysed alongside each of the above samples.

All samples were extracted using water in an ultrasonic bath for 30 minutes. The extracted sample was analysed by liquid chromatography mass spectrometry.

LCMS was performed using the same conditions used to determine free steroids with the exception of the ions monitored.

The ions monitored were:

Oestrone-3-sulfate sodium salt (molecular weight 372.4): 349.36 $[M\text{-}Ma]^-$; 269.2 $[M\text{-}NaSO3]^-$

Oestradiol-3,17-disulfate di-potassium salt (molecular weight 508.7) 469.23 $[M\text{-}K]^-$; 353.59 $[K+Na]^-$

Oestradiol-3-glucoranide-17sulphate (molecular weight 604.8) 527.33 $[M\text{-}2K+H]^-$; 351.36 $[M\text{-}2K\text{-}glucoranidre]^-$.